THE EFFECT OF PHYSOSTIGMINE ON THE RESPONSES OF EARTHWORM BODY WALL PREPARA– TIONS TO SUCCESSIVE STIMULI

E. FRANCES BOTSFORD

(*From the Department of Zoölogy, Connecticut College, and the Marine Biological Laboratory, Woods Hole, Mass.*)

INTRODUCTION

The experiments reported in this paper were performed in an at- tempt to analyze certain augmentation phenomena in the responses of the body wall of the earthworm, *Lumbricus terrestris,* and to provide some basis for an opinion as to their cause.

One type of augmentation concerned in these investigations is that of summation of contraction. When a second stimulus follows the first before the response to the first is completed, the second contraction is superimposed upon the first to produce a greater muscular response. With an increase in the frequency and number of stimuli a condition of tetanus is produced.

Another type of augmentation is shown by the muscles of the body wall when a second stimulation follows the first after the response to the first is completed. In a series of stimulations each successive re- sponse is greater, resulting in a " staircase " effect.

The various augmentation phenomena which are shown by striated, smooth, and cardiac muscle of vertebrates have been studied extensively by many workers. Among the invertebrates, the field is unexplored ex- cept in coelenterates, echinoderms, mollusks, and crustaceans. Because of the diverse neuromuscular mechanisms involved in these different types of invertebrates, the augmentation phenomena themselves present varying characteristics, some being comparable to those found in verte- brates, while others are peculiar to a particular invertebrate group.

The first evidence for summation in the body wall muscles of the earthworm was given by Budington (1902), whose records showed an increase in response corresponding to an increase in the number of shocks administered. After Budington there is no other mention of augmentation phenomena in the earthworm until the studies of Bacq and Coppée (1937), who included three experiments on the earthworm

in their work on *Sipunculus* and the leech. They found that physostig-
mine increased the muscular response when the nerve cord of the earth-
worm was stimulated repetitively.

The purpose of this paper is to show, first, how the muscular re-
sponses of the earthworm body wall are affected by variations in the
frequency and number of single shocks and by the spacing and duration
of tetanic stimulations; second, that the ability of the muscle to give
augmented responses depends upon these time factors; and third, that
the effect of physostigmine upon the muscle responses suggests the par-
ticipation of acetylcholine as a facilitating factor.

An investigation of the muscular responses of the earthworm body
wall is of especial interest since this muscle seems to parallel vertebrate
striated muscle in some of its physiological characteristics. Certain of
these similarities have been referred to by Pantin (1935b) and by Wu
(1939).

Materials and Methods

The specimens of *Lumbricus terrestris* used for these experiments
were kept in an ice-box in moist earth and only the large and healthy
specimens employed.

After partially anesthetizing the earthworm in 0.2 per cent chlore-
tone, a mid-ventral slit was made the length of the worm and the nerve
cord and digestive system were removed. This preparation of the body
wall will be referred to as the muscle strip. At one end it was pinned
to a paraffin block and at the other it was attached to a lever of spring
steel, which recorded the contractions of the longitudinal muscles on a
kymograph drum by a downward deflection of the lever. The approxi-
mate magnification of the lever was five times.

Another preparation used was the whole worm minus about the first
ten segments, arranged for recording muscle contractions in the same
way as described for the muscle strip. This will be referred to as the
whole worm preparation.

For stimulating both the muscle strip and the whole worm, a fine
silver wire electrode leading from a vacuum tube stimulator was in-
serted in each end of the preparation. The stimulator employed a gas
triode 885 arranged to deliver stimuli at frequencies from 1 to 100 per
second. The duration of the bursts of stimuli and the interval between
the bursts were controlled by a commutator in the circuit. Submaximal
stimuli were used in all the experiments described in this paper unless
indicated otherwise.

Results

The Response to Successive Single Shocks

When the body wall preparations were stimulated electrically by a series of submaximal single shocks, the type of response was found to be affected by the frequency of the shocks.

With a frequency of about 2 per second the second response showed an increased contraction, but after that there was no further augmentation. (See Fig. 1, *B*.) With an increase in frequency there was a successive increase in the magnitude of the first four contractions. A small amount of tonus developed which persisted for a short time after the stimulation stopped. (See Fig. 1, *A*.) Increasing the frequency to 6
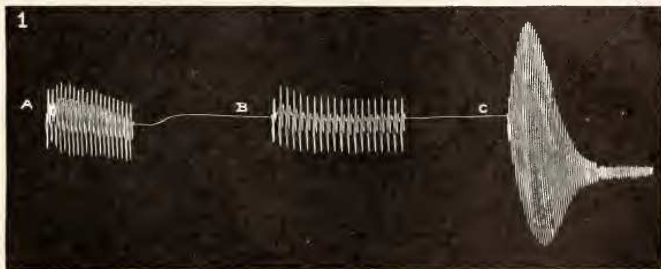

Fig. 1. Effect of frequency of single shocks on augmentation of contractions. Whole worm preparation. Frequency in shocks per second: *A*, 3 per second; *B*, 2 per second; *C*, 6 per second.

per second caused an augmentation in the successive responses up to the ninth, which was nine times greater than the first response. This was followed by an increase in tonus and a diminution in the magnitude of the individual responses. (See Fig. 1, *C*.) The record shows a definite " staircase " effect. Strictly speaking, the term " staircase " should be reserved for the increased muscular responses brought about with maximal stimuli, thus showing that the heightened responses are due to increased contractions of the individual contractile units involved. Since maximal stimuli were not used in these experiments, the descriptive term of " augmentation of responses " is employed, and the determination of the exact mechanism of the facilitating effect is left for future investigation.

With a frequency of 10 per second a state of increased tonus is produced immediately because of the summation of the successive con-

tractions, and with a frequency of about 14 per second, the response shows a condition of completely sustained contraction or tetanus.

Summation of the Responses to a Series of Shocks

This muscle preparation gives a response to a single shock and so can be called a single volley muscle. Records were made on a stationary drum of the responses of the whole worm preparation to one, two, and three single shocks delivered within a 0.3-second period. The response to two shocks was about one and one-half times greater than it was to one, and the response to three shocks was over twice as great.
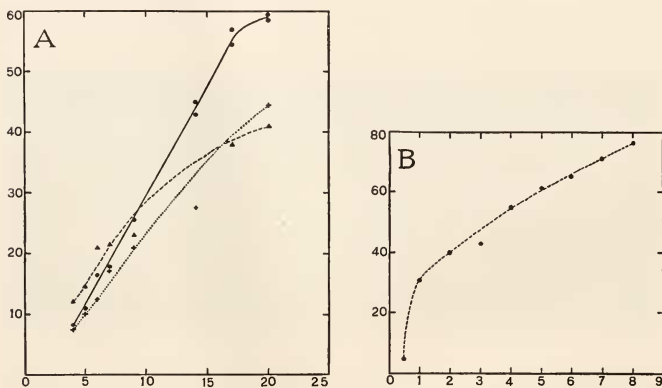

Fig. 2. *A*. Frequency-response curves for muscle strip. Abscissae, number of shocks. Ordinates, response in mm. Duration of bursts, 0.5 second. Interval between bursts, 1 minute. First series shown by dotted line. (Second series omitted.) Third series, unbroken line. Fourth series, broken line. There is a $\frac{3}{4}$ hr. interval between series 3 and 4, a 10-minute interval between the other series.

B. Duration-response curve for whole worm preparation. Abscissae, duration of burst. Ordinates, response in mm. Frequency, 14 shocks per second.

In order to show the effect of frequency upon the magnitude of contraction, a series of responses to a single burst of stimuli was recorded. The duration of every burst was constant, but the frequency of the stimuli within each burst was varied at random over a relatively wide range. A long interval was allowed between the bursts in order to prevent an effect of previous activity upon the response. The responses were recorded on a stationary drum and the length of each measured in millimeters. From the results obtained, frequency-response curves were made. Figure 2, *A* shows three of these curves for one muscle strip.

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The magnitude of the responses increases with each increase in the number of shocks. The increase throughout the first series of trials is nearly in direct proportion to the number of shocks. In the third series the increase in responses is proportional to the number of shocks except with the highest frequency where there is a marked decline in the amount of augmentation. The fourth series was recorded three-quarters of an hour after the third series and about two hours from the beginning of the experiment. Although with the lower frequencies the muscle gives greater contractions than before, with an increase in the number of shocks the amount of augmentation declines, so that with the higher frequencies the responses are lower than in the other two series. This decline is probably due to the deterioration of the preparation.

These results show that the augmentation is proportionally less with the higher frequencies. There is an indication of a slow cumulative building-up process, since the responses of the second series were greater than the first, and those of the third the largest of all.

In one experiment with the whole worm preparation in which the duration of the bursts was gradually increased while the frequency was kept constant, the magnitude of the response to a burst was increasingly greater from the first response measuring 6 mm. up to the tenth measuring 76 mm. A duration-response curve was made by plotting the magnitude of the responses against the duration of each burst. (See Fig. 2, B). Experiments in which the duration of the bursts was changed at random showed the same effect of increased contractions due to longer bursts.

These results show that the magnitude of a response to one isolated burst of stimuli is affected by the frequency of the stimuli within the burst and the duration of the burst.

The Response to Repeated Bursts of Stimuli

When the earthworm body wall is stimulated electrically with shocks at a constant frequency delivered in repeated bursts at appropriate intervals, the first contractions show a definite increase in each successive response. When this augmentation ceases, it is not followed by a plateau, but by an immediate but gradual decline in the magnitude of the successive responses. The rate of decline varies under different conditions of stimulation.

Figure 3 shows the first part of a normal curve for the whole worm preparation. Since the frequency of stimuli within the bursts was 28 per second, these responses were tetanic in character. The first 22 records of contraction show an increase in the response to each successive burst. Figure 4 shows the same phenomenon in a muscle strip

when the same frequency is used. A longer interval was allowed between bursts in the case of the muscle strip preparation because the muscle strip required a longer period to recover its original state of tension after each response.

To investigate the characteristics of the " staircase " effect exhibited by these muscles, many series of responses were recorded showing the
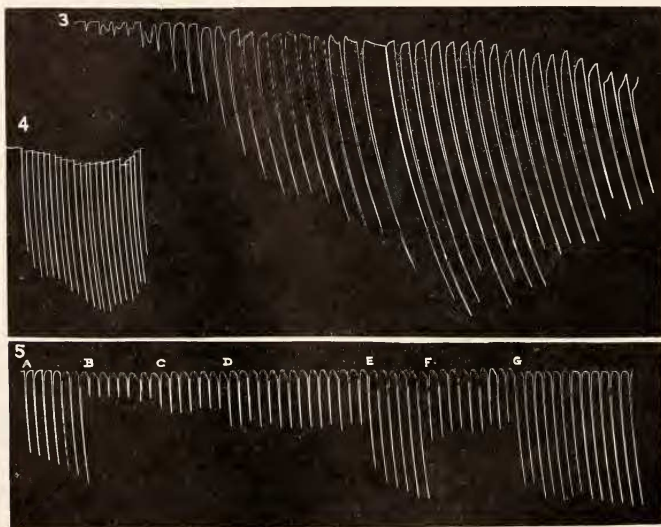

Fig. 3. Augmentation of contractions in whole worm preparation. Duration of burst, 0.3 second. Interval between bursts, 7 seconds. Frequency, 28 shocks per second.

Fig. 4. Augmentation of contractions in muscle strip. Duration of burst, 0.4 second. Interval between bursts, 27 seconds. Frequency, 28 shocks per second. Drum stationary.

Fig. 5. Effect of frequency of stimuli within burst on augmentation of contractions. Whole worm preparation. Duration of burst, 0.3 second. Interval between bursts, 7 seconds. Frequency of stimuli: *A, E, G,* 28 shocks per second causes augmentation; *B,* 9 shocks per second; *C,* 14 shocks per second; *D, F,* 18 shocks per second.

effect upon the augmentation phenomenon of four easily variable conditions: the intensity of the stimulating current, the duration of the bursts of stimuli, the interval between the bursts, and the frequency of the stimuli within the bursts. It should be noted that in some instances it is impossible to make exact quantitative statements which apply to all

the preparations, since the differing physiological states of individual worms produced variation in response.

Difficulties were encountered in working with maximum intensities. In the case of muscle strips, a single burst of stimuli of high intensity induced a condition of tonus which was prolonged to such an extent that successive responses could not be elicited. With the whole worm preparations, high intensities often brought about strong spontaneous contractions which make it impossible to continue with the experiment. In the few successful experiments with high intensities there was no augmentation of the successive responses. This is a crucial point which should be investigated more thoroughly by further experimentation. Because of the disadvantages presented by the use of high intensities, submaximal stimuli of uniform intensity were used in the following experiments.

The length of the interval between bursts has a very definite effect upon the production of augmented responses. Using a frequency of 28 per second with the duration of burst of 0.3 second, there is a striking increase in successive responses in the whole worm preparation when the interval between bursts is 7 seconds. On doubling this interval, there is still some augmentation, but as the interval is increased still more this is less evident, until, with a 30-second interval, the successive responses show no increase.

The duration of the bursts also affects the production of augmented responses. In one experiment in which a frequency of 18 per second was used with bursts spaced at 14-second intervals, the responses were not facilitated when the bursts lasted 0.4 second. Lengthening the bursts to 1.5 seconds built up increasing contractions through six successive responses, the sixth one being more than twice as great as the first.

The frequency of the stimuli within the bursts is a third factor in determining the production of augmented responses. A low frequency does not bring about an increase in the successive responses. Figure 5 shows a long series of responses of the whole worm preparation to bursts of stimuli. The evident arrangement in groups is due to the different frequencies of the stimuli. A frequency of 18 per second or less did not cause augmentation, but with a frequency of 28 per second, three of the groups in the series show facilitated responses. In other preparations, however, increasing contractility was sometimes brought about with lower frequencies.

From the results of these experiments and many others, it is evident that the phenomenon of augmentation is to a great extent dependent on the number of stimuli applied in a unit of time and the grouping of

these stimuli. As the interval between bursts is lengthened, either the
frequency of the stimuli within the bursts must be increased or the
duration of the bursts must be lengthened in order to produce a series
of increasing responses.

The Effect of Physostigmine upon the Production of Augmented Responses

In order to determine the underlying cause of the facilitation phe-
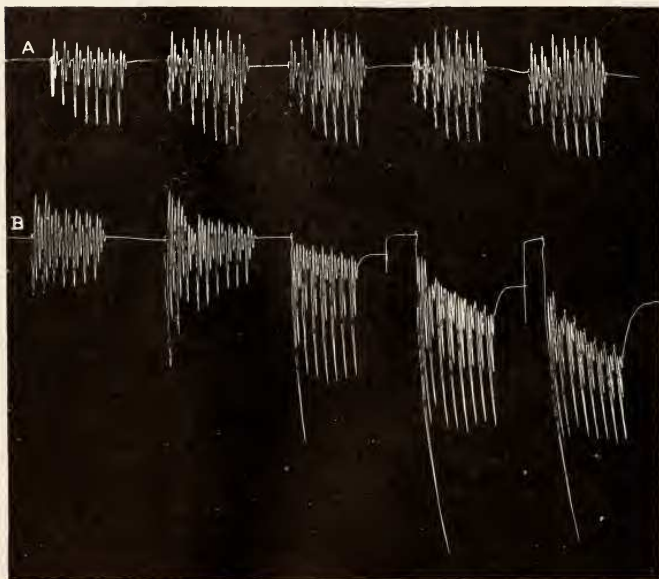nomenon which is manifested in the augmented contractions of the earth-

Fig. 6. Effect of physostigmine 1 gm./10,000 cc. on the response of whole
worm preparation to a series of single shocks. Ten successive series of shocks each
consisting of 7 single shocks in 5 seconds separated from one another by an interval
of 3 minutes. Series A, Ringer's drip. Series B, physostigmine drip.

worm body wall muscles, application of physostigmine was tried. Wu
(1939) has shown that the sensitivity of the body wall to acetylcholine
is greatly increased by physostigmine. From the results of his experi-
ments concerning the action of drugs on the earthworm body wall, he
postulated the presence of some factor which prevented the action of

acetylcholine and which was antagonized by physostigmine. This he thought was probably a high concentration of choline esterase.

If acetylcholine is produced by stimulation of the earthworm muscle preparation, and if this is not completely hydrolyzed by choline esterase before the next stimulation, the persisting acetylcholine could be the cause of the augmentation of the responses. The application of physostigmine which prevents the action of choline esterase could, therefore, show some effect upon the augmentation phenomenon. The following experiments were devised to test this hypothesis.
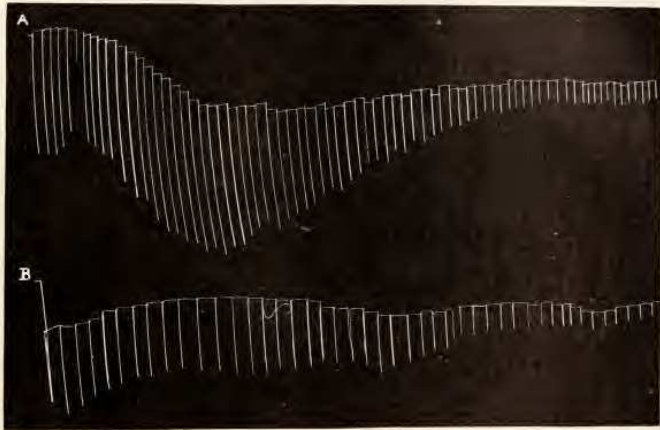

Fig. 7. Effect of physostigmine, 1 gm./10,000 cc., on the responses of muscle strip to a series of bursts of stimuli. Interval between bursts, 27 seconds. Frequency, 40 shocks per second. Drum stationary. *A*, physostigmine applied by drip method during experiment. *B*, muscle strip immersed in physostigmine for 15 minutes previous to stimulation.

The effect of physostigmine on the responses to a series of single shocks was to produce a striking augmentation. The whole worm preparation was stimulated for 5 seconds at a low frequency of 7 shocks in 5 seconds. This was followed by a 3-minute period of rest during which the preparation was given Ringer's solution by drip method. Figure 6, *A* shows a series of five of these 5-second stimulations. Within each of the five groups there is an increase in the successive responses, but the facilitation decays during the 3-minute rest period, so that the first response in each of the five groups is of the same magnitude. Figure 6, *B* shows the result of repeating this procedure except that physostigmine drip 1 gm./10,000 cc. was substituted for the Ringer's

during the 3-minute rest period. Here the first response in each group is increasingly greater. The succeeding responses in one group are smaller than the first response of that group but greater than the normal responses shown in Fig. 6, *A*. A state of maintained tension develops during each 5-second burst of stimuli. The optimum action of the physostigmine, judged by magnitude of response, was reached in $12\frac{1}{4}$ minutes, as shown in the fourth series, in which the maximum response is $8\frac{1}{2}$ times greater than the first response in the normal preparation. The fifth series shows no further increase in magnitude of response.

In order to show the effect of physostigmine upon a series of successive tetanic responses, physostigmine 1 gm./10,000 cc. was applied to a muscle strip preparation continuously by the drip method during a long series of bursts of stimuli. Figure 7, *A* shows the result. A strong tonus was built up gradually in the first 18 responses, but even with this increasing tonus there was an augmentation of the successive individual contractions. On the other hand, when the preparation was

TABLE I

Effect of physostigmine on the magnitude of the responses. Two muscle strips were used: *A*, with physostigmine drip 1 gm./10,000 cc.; *B*, with Ringer's drip. Duration of bursts, 0.3 second. Interval between bursts, 3 minutes. Frequency, 40 shocks per second. The magnitude of response is recorded in the body of the table in mm.

Successive responses	1	2	3	4	5	6	7	8
A. Physostigmine	31	38	45	50	51	53	52	55
B. Ringer's	40	43	43	43	40	40	43	40

put into a bath of physostigmine for 15 minutes before the stimulations began, the first response was of normal magnitude, but following this response an immediate tonus was manifested. The second response was only three-fourths the magnitude of the initial one, and the succeeding responses showed a fatigue-like diminution. The long application of physostigmine prevented any augmentation of contractions. These characteristics can be seen in Fig. 7, *B*. Several control experiments, in which the preparation was left in a bath of Ringer's solution for 15 minutes previous to stimulation, showed a normal response.

Physostigmine was demonstrated to increase the length of the interval between bursts of stimuli which is necessary for the production of augmented responses. As has been stated above, there is a maximum interval of about 30 seconds, which, if exceeded, does not allow augmentation. When a normal muscle preparation is stimulated by bursts which are separated by 3-minute intervals, there is no increase in the successive responses. Table I shows the magnitude of the responses as

measured by the length of the record in millimeters for two muscle strips, one treated with physostigmine drip 1 gm./10,000 cc., the other with Ringer's. When physostigmine drip was used on the preparation, there was an increase in the magnitude of 8 successive responses even though these were separated by 3-minute intervals.

Figure 8 demonstrates this effect in a single muscle strip stimulated in the same way. The first five responses are separated by 3-minute intervals of treatment with Ringer's drip. There is no increase in the successive responses. After the fifth response, treatment with physostigmine drip, 1 gm./10,000 cc., is begun during the 3-minute interval with the result that augmentation is produced as well as an increase in tonus.

The experiments with physostigmine described above suggest that at each burst of stimuli acetylcholine was formed and that the physostigmine acted upon the choline esterase to delay the breakdown of this acetylcholine. This resulted in the persistence of a certain quantity of acetylcholine which caused an increase in the next response of the muscle.

It will be noted that a relatively high concentration of physostigmine was used in these experiments. This was in order to favor the diffusion of sufficient drug into the tissues to produce an immediate effect even with the slow drip method employed.

Further indication of the formation of some facilitating substance at the time of stimulation is shown by conditions of tonus succeeding the muscular responses. Figure 9 shows a response in which the primary contraction and partial relaxation due to the single shock is followed by a smaller and slower contraction and relaxation. In Figure 10 is recorded the response to a series of 7 single shocks after the preparation had been in physostigmine 1 gm./100,000 cc. for 4 minutes. After the seventh response, the stimulation having stopped, there is a long slow contraction followed by relaxation.

The persistence of a facilitating effect is demonstrated in Fig. 11. Here a series of augmented responses was produced using a frequency of 28 per second. This was followed, with no break in the intervals of stimulation, by a series of bursts with a frequency of 9 per second, which is not a facilitating frequency. The first four responses to the low frequency bursts show a greater response than those which follow. The preceding series of augmented responses had a facilitating effect which lasted 29 seconds. This corresponds to the interval of 30 seconds determined to be the maximum interval within which facilitation can occur. The response to a single shock is also increased when immediately preceded by a series of augmented responses.
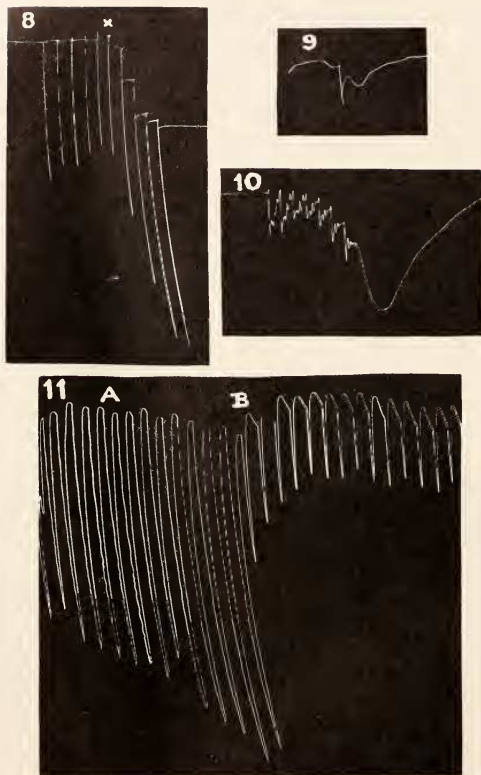
FIG. 8. Effect of physostigmine 1 gm./10,000 cc. in producing augmentation of contraction in a muscle strip with an interval of 3 minutes between bursts of stimuli. X represents physostigmine drip begun. Duration of bursts, 0.3 second. Frequency, 40 shocks per second.

FIG. 9. Response of muscle strip to a single shock followed by a change in tonus.

FIG. 10. Responses of muscle strip to a series of 7 single shocks after 4 minutes in physostigmine 1 gm./100,000 cc. Following the responses there is a change in tonus.

FIG. 11. Increased magnitude of the initial responses to bursts of low frequency when preceded by a series of successively augmented responses. Whole worm preparation. Frequency of stimuli: A, 28 shocks per second; B, 9 shocks per second. Duration of burst, 0.3 second. Interval between bursts, 7 seconds.

Discussion

From the results of the experiments described it is evident that previous activity has a definite facilitating effect upon subsequent contractions of the longitudinal muscles in the body wall of the earthworm.

When preparations are stimulated with successive single shocks, three types of responses are possible, depending upon the frequency of the shocks: first, an increase in magnitude of successive separate contractions; second, a partial summation of the successive contractions; and third, complete tetanus. Since, with increase in frequency of shocks, the first type of response merges gradually into the second, and the second into the third, it would appear that the same facilitating factor is responsible for all. This is also indicated by the fact that with the application of physostigmine it is possible to produce a summation of contractions or tetanus with the lower frequencies.

In his work on Actinozoa, Pantin (1935a) found that in different neuromuscular mechanisms he was able to demonstrate these three types of responses depending upon the time relation between the duration of a complete contraction and relaxation of the muscle, and the duration of the persistence of the facilitating factor.

In the earthworm preparations the facilitating factor persists between 0.4 and 0.5 second after a single shock. With repeated shocks the facilitation effect is cumulative, resulting in considerably heightened contractions, as exhibited by the responses to repeated tetanic stimulations. Under these conditions the facilitating factor may last nearly 30 seconds, as shown by an increased response to a second tetanic stimulation within that period of time.

This phenomenon of facilitation has been demonstrated in the muscular reactions of other invertebrates. The number and frequency of stimuli are the decisive factors in producing the augmented responses, as is the case in the earthworm, but the cause of the facilitation seems to be different in the various neuromuscular mechanisms.

Pantin (1935a), in his work on facilitation in Actinozoa, came to the conclusion that in certain muscles the increasing magnitude of the responses in a series of stimuli is due to neuromuscular facilitation, by which, with each succeeding stimulus more muscle fibers are affected. He found no evidence at that time for the functioning of chemical mediators in coelenterates. Ross and Pantin (1940), in their investigation of the effect of certain ions on facilitation in Actinozoa, found that two factors were involved in facilitation. Although they did not determine the nature of the facilitating process they concluded that it could not be due solely to a transmitter.

In crustacean striated muscle, according to Katz (1936), the frequency to which individual muscle fibers respond varies, so that the number of fibers which contract and consequently the magnitude of the contraction, are controlled by the frequency of the nerve impulses.

In the present paper evidence has been offered to support the view that in the earthworm body wall the facilitating factor is acetylcholine. The effect of physostigmine in producing greatly augmented responses points to this. The most convincing evidence is the ability of physostigmine to delay the decay of the facilitating factor, so that augmented responses are elicited even with long intervals between tetanic stimulations.

The location of this facilitating effect is a subject for further investigation. Since the phenomenon showed the same characteristics in the muscle strip as in the whole worm preparation, it cannot be dependent on the nerve cord.

SUMMARY

1. The longitudinal muscles of the body wall of the earthworm (*Lumbricus terrestris*) show augmented responses when stimulated by successive single shocks at low frequencies. The facilitating condition lasts not more than 0.5 second after the response to a single shock.

2. A frequency of 14 per second results in a complete summation of the contractions or a condition of tetanus.

3. The magnitude of a summated response elicited by a series of shocks is proportional to the duration of the burst of shocks and to the frequency of the shocks within the burst.

4. The tetanic responses to repeated bursts of stimuli show an increasing augmentation of the initial contractions. The production of this "staircase" effect is affected by the frequency of the shocks, the duration of the bursts, and the length of the interval between bursts.

5. After a brief tetanus the facilitating condition persists for nearly 30 seconds.

6. The application of physostigmine increases the augmentation of responses and tends to produce a condition of tonus.

7. Physostigmine delays the decay of the facilitating property so that augmented responses are produced with intervals as long as 3 minutes between bursts of stimuli, suggesting the rôle of acetylcholine in the production of the augmented responses.

ACKNOWLEDGMENTS

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